Antonie van Leeuwenhoek **77:** 223–228, 2000.

Saccharomyces bulderi sp. nov., a yeast that ferments gluconolactone

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Received 16 July 1999; accepted 8 October 1999

Key words: fermentation, gluconolactone, raffinose, *Saccharomyces bulderi*, yeast

Abstract

An unknown yeast species was isolated from maize silage and was determined to be novel on the basis of morphological and physiological characteristics, nucleotide sequence of domain D1/D2 of LSU rDNA and from its electrophoretic karyotype. The name for the proposed new species is *Saccharomyces bulderi* Middelhoven, Kurtzman et Vaughan-Martini (type strain CBS 8638, NRRL Y-27203, DBVPG 7127). *S. bulderi* is closely related to *S. barnettii* and *S. exiguus* from which it can be distinguished by having a double vitamin requirement of biotin and thiamine and by no or slow aerobic growth on raffinose, a sugar that on the contrary is fermented rapidly. Gluconolactone is rapidly fermented with ethanol, glycerol and carbon dioxide being the main products.

Introduction

Analysis of the yeast flora of maize silage revealed a predominance of *Candida* spp. and of two *Saccharomyces* spp. which were identified as *S. dairenensis* and *S. exiguus* (Middelhoven & Franzen 1986). Recently, maize ensiled in strictly anaerobic conditions at 20 °C was found to be inhabited by an unknown yeast species. In the present paper this species is characterized from phenotype, nucleotide divergence in domain D1/D2 of LSU rDNA, and from electrokaryotype. The data suggest that the species is unknown and the name *Saccharomyces bulderi* Middelhoven, Kurtzman et Vaughan-Martini is proposed.

Methods and Materials

Isolation of the strain

Dough-state whole-crop chopped maize was ensiled in a tightly sealed jar. After 3 months at 20 °C 0.1 ml of a 1/10 dilution in sterile water was plated on Difco YMA supplemented with 25 mg each of streptomycin and tetracyclin per litre. After 2 days incubation at 25 °C 37 colonies of two different size types appeared, of 5 and 2 mm diameter, respectively, in about equal numbers. Three colonies of each type were streaked pure and were maintained on YMA slants.

Characterization of the strains

The isolates were examined for morphological and physiological properties using standard yeast identification methods (i.e. Van der Walt & Yarrow 1984). Utilization of carbon and nitrogen sources in liquid Difco Yeast Nitrogen Base (YNB) and Yeast Carbon Base was examined at 25 °C on a rotary shaker at a speed of 100 rpm, with 2.5 ml of growth medium in culture tubes of 17 mm width. Utilization of nitrite was confirmed by the auxanographic technique. The pH of growth media was adjusted to 5.5 if required, but media with galacturonic or quinic acid or potassium *hemi*-saccharate were not neutralized, which is in agreement with the laboratory practice of CBS Delft (D.Yarrow, pers. comm.; cf. Middelhoven 1997). Fermentation of sugars was studied in Durham tubes supplied with 1% yeast extract or YNB and 2%

of the sugar studied. Ascospores were observed after growing the strains on McClary's acetate agar (0.1% glucose, 0.18% potassium chloride, 0.82% sodium acetate trihydrate, 0.25% yeast extract and 1.5% agar) for 7 days at 30 °C followed by 3 weeks at 5 °C.

Sequence analysis, determination of electrophoretic karyotype and of mol%G+C

The nucleotide region examined was the ca. 600 base-pair D1/D2 domain at the 5′ end of the 26S rDNA, and methods for sequencing and sequence analysis were reported (Kurtzman & Robnett 1998). The electrophoretic karyotype was determined by pulsed field gel electrophoresis (PFGE) using a CHEF Mapper (Bio-Rad, Hercules, CA, USA). Sample and gel preparation were as described by Vaughan-Martini (1995) and electrophoresis parameters were as described in Figure 2. High-molecular weight nuclear DNA was prepared according to Vaughan-Martini (1995). The mol%G+C was determined on the basis of the thermal denaturation temperature (Tm) obtained using the technique of Marmur and Doty (1962). Experiments were performed on a Beckman DU 650 Spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA) equipped with a Beckman High Performance Temperature Controller. Samples containing 25 μg/ml of DNA were analyzed against a control DNA of the type strain of *Candida parapsilosis* (CBS 604). The mol%G+C was calculated from the mean Tm value obtained from the average of at least 3 analyses.

Analysis of fermentation products

The HPLC analysis of culture broth was according to van Hoek et al. (1998)

Results

Latin diagnosis of *Saccharomyces bulderi* Middelhoven, Kurtzman et Vaughan-Martini sp.nov.

In medio liquido dextrosum et peptonum et extractum levidinis continente post 3 dies ad 20 °C cellulae globosae et ovoideae (2.7−3.9 × 3.3−4.4 Fm), singulae vel binae. Sedimentum adest. Etiam post 4 hebdomades sedimentum adsunt. Cultura in agaro extracta malti et levidinis continente post 3 dies ad 25 °C cremea, haud lucida, butyrosa, post hebdomades 4, 20 °C, eadem forma. In agaro PDA dicto post dies 3, 20 °C, neque mycelium neque pseudomycelium formatur. Asci formantur ex transformatione cellularum vegetativarum diploidearum. Ascosporae glabrae, rotundae aut ovoideae; 1–2 in asco. D-Glucosum, D-galactosum, sucrosum, trehalosum, raffinosum, gluconolactonum fermentantur neque maltosum, melibiosum, lactosum, cellobiosum, melezitosum, inulinum et acidum gluconicum. D-Glucosum, D-galactosum, sucrosum, trehalosum, raffinosum (lente) et gluconolactonum assimilantur. Sorbosum, D-glucosaminum, acetyl-D-glucosaminum, D-ribosum, D-xylosum, L-arabinosum, D-arabinosum, L-rhamnosum, maltosum, α-methyl-D-glucosidum, cellobiosum, salicinum, arbutinum, melibiosum, lactosum, melezitosum, inulinum, amylum solubile, glycerolum, erythritolum, xylitolum, L-arabitolum, D-glucitolum, D-mannitolum, galactitolum, inositolum, acidum 2-keto-D-gluconicum, acidum 5-keto-D-gluconicum, acidum gluconicum, acidum glucuronicum, acidum galacturonicum, acidum lacticum, acidum succinicum, acidum citricum, methanolum, ethanolum, propano-1,2-diolum, butano-2,3-diolum, acidum quini
cum, acidum saccharicum et acidum galactonicum non assimilantur. Kalii nitratum, sodii nitritum, ethylaminum, L-lysinum, cadaverinum, creatinum, creatininum, glucosaminum et imidazolum non assimilantur. Thiaminum et biotinum externa ad crescentiam necessaria sunt. 31 °C crescit neque 37 °C. Crescere potest 0.1 g/litrum cycloheximidi neque 1 g/litrum. Ureum non finditur. Materia amyloidea non formatur. Mol% G+C 33.5. Typus CBS 8638^{T} isolatus e Zea mayde acidificata, in collectione zymotica Centraalbureau voor Schimmelcultures, Delft, The Netherlands.

Morphological characteristics of the species

After 3 days of growth in glucose (2%, wt/vol), yeast extract (0.5%, wt/vol), peptone (1.0%, wt/vol) broth at 25 °C the cells are globose and ovate, 2.7–3.9 × 3.3–4.4 Fm. Budding yeast cells are present. A sediment but no pellicle is formed. After 4 weeks a sediment is still present. The slant culture on YM agar after 3 days at 25 °C is scant, cream, butyrous and dull. In the slide culture on potato dextrose agar (PDA) after 3 days at 25 °C only ovate budding cells are formed. Asci containing 1–2 globose to ovate ascospores are formed, in very low amounts (<1%), on acetate agar slants after 1 week at 30 °C followed by 3 weeks at 5 °C (Figure 1).

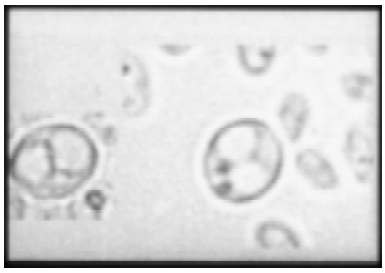

Figure 1. Ascosporogenous culture of *Saccharomyces bulderi* CBS 8638^{T}.

Table 1. Assimilation of carbon compounds by *Saccharomyces bulderi* CBS 8638

D-Glucose	+	Ribitol	–
D-Galactose	+	Xylitol	–
L-Sorbose	–	L-Arabinitol	–
D-Glucosamine	–	D-Glucitol	–
D-Ribose	–	D-Mannitol	–
D-Xylose	–	Galactitol	–
L-Arabinose	–	*myo*-Inositol	–
D-Arabinose	–	D-Glucono-1,5-lactone	+
L-Rhamnose	–	2-Keto-D-gluconate	–
Sucrose	+	5-Keto-D-gluconate	–
Maltose	–	D-gluconate	–
$\alpha\alpha$-Trehalose	+	D-glucuronate	–
Methyl-α-D-glucoside	–	D-Galacturonic acid	–
Cellobiose	–	DL-Lactate	–
Salicin	–	Succinate	–
Arbutin	–	Citrate	–
Melibiose	–	Methanol	–
Lactose	–	Ethanol	–
Raffinose	D,–	Propane-1,2-diol	–
Melezitose	–	Butane-2,3-diol	–
Inulin	–	Quinic acid	–
Soluble starch	–	*hemi*-Saccharate	–
Glycerol	–	Galactonate	–
Erythritol	–	Acetyl-D-glucosamine	–

Table 2. Assimilation of nitrogenous compounds by *Saccharomyces bulderi* CBS 8638

Nitrate	–	Creatine	–
Nitrite	–	Creatinine	–
Ethylamine	–	Glucosamine	–
L-Lysine	–	Imidazole	–
Cadaverine	–		

Alcohol fermentation

Gas is formed during fermentation of 2% glucose, galactose, sucrose, trehalose, raffinose and gluconolactone, dissolved in 1% yeast extract or in YNB, but not from maltose, melibiose, lactose, cellobiose, melezitose, inulin, soluble starch and D-xylose. In addition to large amounts of ethanol, considerable amounts of glycerol were detected in the fermentation broth of 2% gluconolactone in YNB (Table 3).

Assimilation of carbon and nitrogen compounds

Growth responses on standard carbon and nitrogen compounds are shown in Tables 1 and 2, respectively.

Other characteristics

Vitamin requirements are met by thiamine plus biotin. Growth occurs at 31 °C but not at 37 °C, nor in 50% glucose yeast extract agar or in the presence of 10% sodium chloride. Growth in the presence of 0.1% cycloheximide was absent, but 0.01% of this antibiotic was tolerated. Urea was not hydrolyzed. The colour reaction with DBB was negative. Amyloid compounds were not formed. Mol% G+C 33.5.

Sequence analysis of the large subunit rDNA

The nucleotide sequence of the D1/D2 domain of the 26S rDNA for CBS 8638 was deposited in GenBank under the accession number AF125391. Those of the closely related *S. barnettii* Vaughan-Martini in GenBank under the accession number U84231. The nucleotide sequences for domain D1/D2 of CBS 8638, CBS 8639 and NRRL Y-27205 were identical and differed from *S. barnettii*, the closest relative, at just three positions. On the basis of the phylogenetic analysis of Kurtzman & Robnett (1998), *S. bulderi* is a member of the clade comprising *S. barnettii, S. exiguus* and *Candida humilis*.

Electrophoretic karyotypes

As seen in Figure 2, strains CBS 8368 and 8369 and DBVPG 7128 show relatively similar electrophoretic karyotypes which are distinctly different from the type strains of closely related species *C. milleri* and *S. barnettii* (lanes 2 and 7). On the other hand, the patterns of all three strains of *S. bulderi* appear to have some similarity to that of the type strain of *S. exiguus* (lane 3), although the latter has chromosomal bands

Table 3. Fermentation products of *S. bulderi* CBS 8638 after growth in 2% gluconolactone in YNB for 7 days in a Durham tube

Ethanol	143 mM
Glycerol	17 mM
Lactic acid	3.0 mM
Acetic acid	1.5 mM
Pyruvic acid	0.5 mM
Succinic acid	1.0 mM

both above 1250 kb and below 680 kb which were not seen in the *S. bulderi* strains. While strains CBS 8638 and DBVPG 7128 have nearly identical electrophoretic karyotypes, strain DBVPG 7129 shows some variations in both the large (over 1,000 kb) and medium (500–1,000 kb) ranges. Nevertheless, considering that all three strains have shown identical D1/D2 sequences (this study), it appears evident that these slight differences are simply the expression of interspecific variability allready seen in the *S. exiguus* complex (Vaughan-Martini 1995).

Origin and deposits

The strains had been isolated from ensiled whole-crop maize in Jauary 1998. The type strain *Saccharomyces bulderi* Middelhoven, Kurtzman et Vaughan-Martini CBS 8638^T has been deposited in the yeast collection of the Centraalbureau voor Schimmelcultures, Delft, The Netherlands. Strain DBVG 7129 is physiologically identical to the type strain, but has a slightly deviating karyotype (Figure 2). The accession numbers of *S. bulderi* strains in other culture collections are DBVPG 7127–7129, resp. and NRRL Y–27203-27205, resp.

Etymology

The specific epithet *bulderi* is in honour of professor Dr C.J.E.A. Bulder who studied respiratory deficiency in yeasts from a systematics and physiological viewpoint (Bulder 1963, 1964, 1966).

Discussion

The yeast flora of a 3-month-old anaerobic maize silage consisted of two species which were present in about equal numbers (appr. 2.000 CFU per gram). One species could be identified as *Saccharomyces exiguus* Reess ex E.C. Hansen; the other one, characterized by smaller colonies, could not be identified. Sequencing of the D1/D2 region of the 26S rDNA subunit revealed a close relationship to *S. barnettii* Vaughan-Martini (1995), with only three base pairs difference. Because of the relatively small nucleotide divergence, additional data are required to demonstrate that the proposed species is not a divergent form of *S. barnettii*. As seen in Figure 2, the comparative determination of the electrophoretic karyotypes of the three *S. bulderi* strains against the type strains of other closely related species has shown a good similarity between the strains under study. On the other hand, these profiles were significantly different from those obtained for *C. milleri* and *S. barnettii* and showed at least three bands less than those seen for the type strain of *S. exiguus*. *S. bulderi* can be distinguished from the closely related *S. barnettii* by its higher maximum growth temperature (31 °C), and from *S. barnettii* and *S. exiguus* by its double vitamin requirement, both biotin and thiamine being necessary, and by slow or absent aerobic growth on raffinose, a sugar that is fermented rapidly.

As compared to most other *Saccharomyces* strains, growth of *S. bulderi* on conventional agar slants was scant. *S. bulderi* is characterized by failure to assimilate non-fermentable substrates such as ethanol and lactic acid, even at pH 4.0 and in the presence of yeast extract, conditions that have been proven to stimulate growth on these substrates of yeast strains isolated from maize silage (Middelhoven & Franzen 1986). Respiratory deficiency of *S. bulderi* could have been the cause of this failure, but rapid fermentation of galactose and the production of asci argue against this hypothesis. Petite mutants of *S. cerevisiae* do not ferment or assimilate galactose and are unable to form ascospores.

Growth of all 3 strains of *S. bulderi* on some sugars was faster under semi-anaerobic conditions, *viz.* in the Durham tube, than under aerobic conditions as applied in this study. This was especially the case with raffinose. This trisaccharide was fermented in the Durham tube within 2 days, while aerobic growth on this sugar was absent or very delayed. This was not an effect of the growth medium. Replacing the standard 1% yeast extract in the Durham tube with YNB gave the same rapid responses. Aerobic growth on raffinose in the presence of yeast extract was also absent or very slow. Fermentation of trehalose and gluconolactone was also faster than their aerobic assimilation.

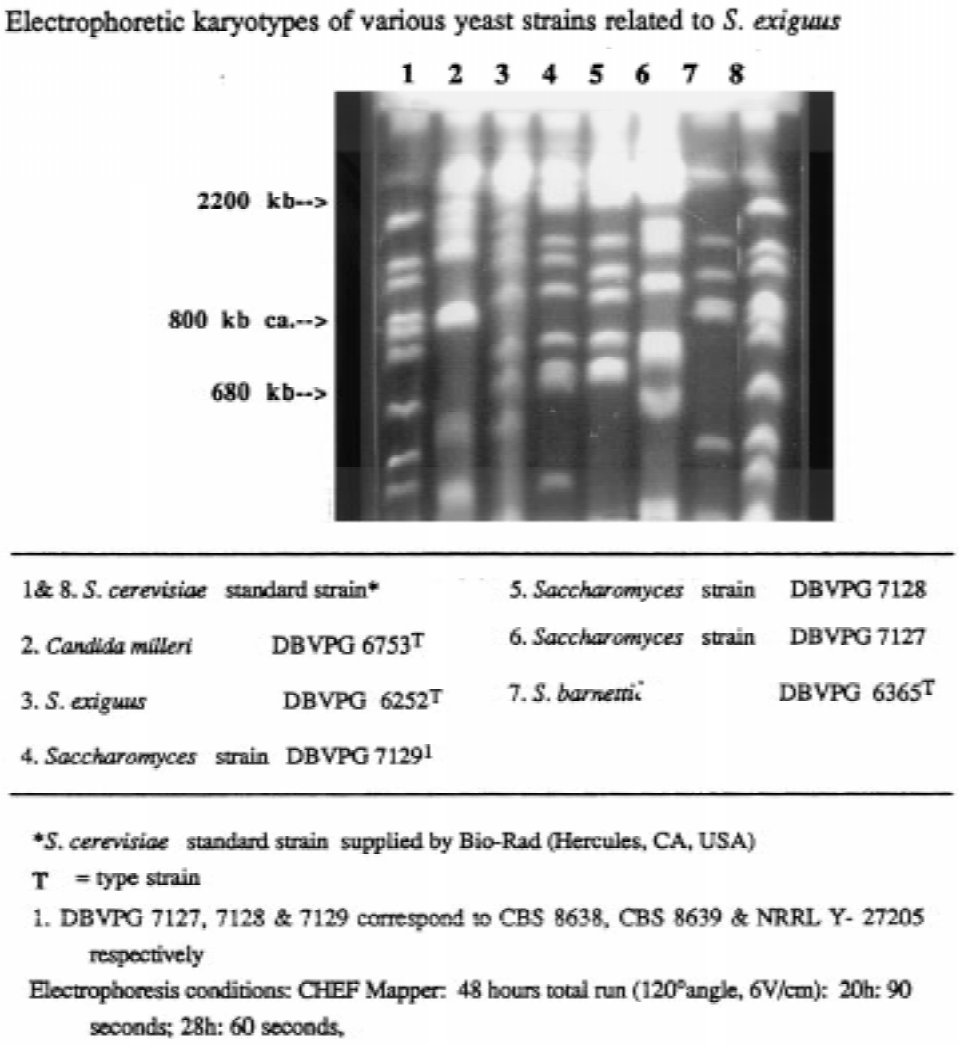

Figure 2. Electrophoretic karyotypes of various yeast strains related to *S. exiguus*.

On the contrary, when glucose, galactose and sucrose were provided as carbon sources, the favourable effects of semi-anaerobiosis on the growth rate were not observed. Fermentation of sugars that are not assimilated aerobically is very unusual. It has generally been believed to be impossible (Kluyver 1914).

In view of the failure of *S. bulderi* to assimilate non-fermentable substrates, growth on and fermentation of gluconolactone were unexpected. Analysis of the fermentation broth revealed presence of ethanol and of relatively large amounts of glycerol. The latter is a common side product of the alcoholic fermentation of sugars by yeasts. Its production serves to restore the redox balance that is disturbed due to biomass production. Biomass is more oxidized than carbohydrates, resulting in an excess of NADH which under anaerobic conditions cannot be removed by oxidation. As gluconolactone is also more oxidized than carbohydrates, the presence of glycerol among the fermentation products was not expected. On the contrary, production of fermentation products more oxidized than ethanol, e.g. acetate, would have been more likely. Acetate, however, is produced only in trace amounts (Table 3). A more detailed physiological study will be carried out in order to throw some light upon this apparent controversy.

Acknowledgements

Thanks are due to Dr Stefanie Oude Elferink for supplying the sample of maize silage, to Dr Jack T. Pronk and Mrs. Marijke Luttik for carrying out the HPLC analyses, to Ms Stefania Barcaccia for doing the Tm and PFGE analyses, to Dr W. Gams for correcting the Latin diagnosis and to Christie J. Robnett for determining the D1/D2 domain nucleotide sequences for strains of *S. bulderi*. One of us (AV-M) thanks Progetto Coordinato CNR: Biodiversità microbica in ambienti agro-industriali, Sigla 'Germic' Codice 6623 for financial support.

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